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Description

POLYNUCLEOTIDE ENCODING 2-HYDROXYISOFLAVANONE DEHYDRATASE AND USE THEREOF

[Technical Field]

The present invention relates to a 2-hydroxyisoflavanone dehydratase that catalyzes a dehydration reaction of 2-hydroxy isoflvanone, novel polynucleotides encoding it, and uses thereof.

[Background Art]

Isoflavones and compounds derived therefrom (i.e., isoflavonoids) are components unique to leguminous plants and have attracted attention as a health supplement in recent years. In addition, isoflavonoids including isoflavones have been known to play a very important role as antimicrobial agents and symbiotic signals for the plants to adapt to biological environments.

The simplest skeletal structure of isoflavonoid is isoflavone, which is one of a group of isoflavonoids, produced early by flavanoid metabolism (see Fig. 1). Isoflavones and their glycosides are accumulated in organs of leguminous plants. Daidzein (7,4'-dihydroxyisoflavone) and genistein (5,7,4'-trihydroxyisoflavone) contained in the free forms and in the form of glycosides in soybean seeds have been known as phytoesterogen (plant esterogen) for the promotion of the health

of humans and for the prevention of diseases.

Isoflavone is an intermediate product in the biosynthesis of isoflavonoids having ecophysiological activity, such as antimicrobial phytoalexins having a pterocarpan or isoflavan skeleton. Approximately 50% of isoflavonoids have functional groups derived from 4′-methoxyl group, and these compounds are mainly derived from 4'-methoxylated isoflavone, formononetin (7-hydroxy-4'-methoxyisoflavone).

The isoflavonoid skeleton is biosynthetically produced from (*2S*)-flavanone by the action of a cytochrome P450 (P450), i.e., 2-hydroxyisoflavanone synthase (IFS). The IFS catalyzes the hydroxylation of the carbon at position 2 of the flavonoid skeleton with the rearrangement of 1,2-aryl group. The resulting product, 2-hydroxyisoflavanone, is dehydrated to form isoflavone (see Fig. 1).

cDNAs of IFS have been identified in one of the leguminous plants, *Glycyrrhiza echinata* (hereinafter, referred to as "licorice") (Non-Patent Document 1 and Patent Document 1) and soybean (Non-Patent Document 2 and Non-Patent Document 3). In the *in vitro* assay using recombinant IFS which had been overexpressed in yeast microsomes, a large amount of isoflavone was produced by spontanious dehydration of the initial product, 2-hydroxyisoflavanone, in addition to the initial product (Non-Patent Document 1 and Non-Patent Document 3). Furthermore, it was reported that IFS expressed in

insect cells produced only isoflavone (Non-Patent Document 2). In this way, an isoflavone can be produced non-enzymatically from the direct product of IFS reaction. In addition, (*2S*)-flavanone, the substrate of IFS, is a common component present in both leguminous and non-leguminous plants. Thus, it was expected that a non-leguminous plant containing no isoflavonoids could be transformed to the plant having an ability of producing isoflavones by using the IFS cDNA (Non-Patent Document 4, Non-Patent Document 5, and Non-Patent Document 6).

On the basis of these findings, attempts have been made to produce isoflavone in non-leguminous plants (*Arabidopsis thaliana* and tobacco), which inherently contain no isoflavone, by introduction of a soybean IFS gene. However, the production was as low as around 1/1,000 of that by soybean seeds (Non-Patent Document 3, Non-Patent Document 7, and Non-Patent Document 8). Therefore, it is considered that IFS alone cannot perform the production of isoflavone in an efficient manner.

On the other hand, the enzymatic activity of 2-hydroxyisoflavanone dehydratase, which converts 2,7,4′-trihydroxyisoflavanone into daidzein, was detected in cells of *Pueraria lobata* (kuzu beans) and the protein thereof was then purified (Non-Patent Document 9 and Non-Patent Document 10). In addition, in experiments conducted by the inventors of the present invention, 2,7-dihydroxy-4′-methoxyisoflavanone was converted

into formononetin in licorice cell-free extract but 2,7,4′-trihydroxyisoflavanone was not converted into daidzein (Non-Patent Document 11). These results indicate that the dehydration of 2-hydroxyisoflavanone to isoflavone in plant cells may depend on an enzyme (i.e.,2-hydroxyisoflavaone dehydratase) and the enzyme may have substrate specificity for 2-hydroxyisoflavanones having different substituents.

In this way, the previous studies have revealed that isoflavone cannot be efficiently produced only by IFS and the substrate-specific enzymes play an important role in the dehydration of 2-hydroxyisoflavanones to isoflavones. However, the details of the enzyme (2-hydroxyisoflavanone dehydratase) which contributes to the dehydration of 2-hydroxyisoflavanone, to isoflavones have not been elucidated.

[Patent Document 1]

WO 00/46356 pamphlet

[Non-Patent Document 1]

Akashi, T., Aoki, T. and Ayabe, S. (1999) Cloning and functional expression of a cytochrome P450 cDNA encoding 2-hydroxyisoflavanone synthase involved in biosynthesis of the isoflavonoid skeleton in licorice. Plant Physiol. 121: 821-828.

[Non-Patent Document 2]

Steele, C.L., Gijzen, M., Qutob, D. and Dixon, R.A. (1999) Molecular characterization of the enzyme catalyzing the aryl migration

reaction of isoflavonoid biosynthesis in soybean. Arch. Biochem. Biophys. 367: 146-150.

[Non-Patent Document 3]

Jung, W., Yu, O., Lau, S. M., O'Keefe, D. P., Odell, J., Fader, G. and McGonigle, B. (2000) Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. Nature Biotechnol. 18: 208-212.

[Non-Patent Document 4]

Dixon, R.A. and Steele, C.L. (1999) Flavonoids and isoflavonoids - a gold mine for metabolic engineering. Trends Plant Sci. 4: 394-400.

[Non-Patent Document 5]

Humphreys, J. M. and Chapple, C. (2000) Molecular 'pharming' with plant P450s. Trends Plant Sci. 5: 271-272.

[Non-Patent Document 6]

Feldmann, K.A. (2001) Cytochrome P450s as genes for crop improvement. Curr. Opin. Plant Biol. 4: 162-167.

[Non-Patent Document 7]

Yu, O., Jung, W., Shi, J., Croes, R.A., Fader, G.M., McGonigle, B. and Odell, J.T. (2000) Production of the isoflavones genistein and daidzein in non-legume dicot and monocot tissues. Plant Physiol. 124: 781-793.

[Non-Patent Document 8]

Liu, C.J., Blount, J.W., Steele, C.L. and Dixon, R.A. (2002) Bottlenecks for metabolic engineering of isoflavone glycoconjugates in Arabidopsis. Proc. Natl. Acad. Sci. USA 99: 14578-1458 3.

[Non-Patent Document 9]

Sankawa, U. and Hakamatsuka, T. (1997) Biosynthesis of isoflavone and related compounds in tissue cultures of *Pueraria lobata*. In Dynamic aspects of natural products chemistry. Molecular biological approaches. Edited by Ogura, K. and Sankawa, U. pp. 25-48. Kodansha/Harwood Academic, Tokyo.

[Non-Patent Document 10]

Hakamatsuka, T., Mori, K., Ishida, S., Ebizuka, Y. and Sankawa, U. (1998) Purification of 2-hydroxyisoflavanone dehydratase from the cell cultures of *Pueraria lobata*. Phytochemistry 49: 497-505.

[Non-Patent Document 11]

Akashi, T., Sawada, Y., Aoki, T. and Ayabe, S. (2000) New scheme of the biosynthesis of formononetin involving 2,7,4′-trihydroxyisoflavanone but not daidzein as the methyl acceptor. Biosci. Biotechnol. Biochem. 64:2276-2279.

[Non-Patent Document 12]

Akashi, T., Sawada, Y., Shimada, N., Sakurai, N., Aoki, T. and Ayabe, S. (2003) cDNA cloning and biochemicalcharacterization of *S*-adenosyl-L-methionine:2,7,4′-trihydroxyisoflavanone

4′-*O*-methyltransferase, a critical enzyme of the legume isoflavonoid phytoalexin pathway. Plant Cell Physiol. 44:103-112.

[Non-Patent Document 13]

Ayabe, S., Akashi, T. and Aoki, T. (2002) Cloning of cDNAs encoding P450s in the flavonoid/isoflavonoid pathway from elicited leguminous cell cultures. Methods Enzymol. 357: 360-369.

[Non-Patent Document 14]

Nakamura, K., Akashi, T., Aoki, T., Kawaguchi, K. and Ayabe, S. (1999). Induction of isoflavonoid and retrochalcone branches of the flavonoid pathway in cultured *Glycyrrhiza echinata* cells treated with yeast extract. Biosci. Biotechnol. Biochem. 63: 1618-1620.

[Disclosure of the Invention]

The present invention is intended to isolate a dehydratase that plays an important role in the process of producing isoflavone in plant cells and to find out the amino acid sequence thereof and a nucleotide sequence encoding the amino acid sequence.

More specifically, the present invention is intended to determine the amino acid structure of 2-hydroxyisoflavanone dehydratase that catalyzes the dehydration of 2-hydroxyisoflavanone to isoflavone and thereby provide a gene encoding the amino acid structure. Furthermore, the present invention is intended to use the gene thus obtained for the production of isoflavonoid including isoflavone.

For solving these problems, at first, the inventors of the

present invention have found the presence of a species-specific 2-hydroxyisoflavanone dehydratases through the investigation of the dehydratase activities in both licorice (formononetin-producing plant) and soybean (daidzein-producing plant) extracts. Then, a cDNA that encodes 2,7-dihydroxy-4′-methoxyisoflavanone-2,3-dehydratase (formononetin-synthetic enzyme) was isolated from licorice by the use of an advanced gene-cloning method named "Function expression Fractionation Screening" (Non-Patent Document No. 12). Furthermore, using the sequence information obtained as above, another cDNA of an analogous enzyme having a different substrate specificity, i.e., 2,7,4′-trihydroxyisoflavanone-2,3-dehydratase (daidzein-synthesizing enzyme) of soybean was obtained.

The novel gene that encodes 2-hydroxyisoflavanone dehydratase may allow for the increasing production of isoflavonoid in a non-leguminous plant by introduction of the gene in the plant.

In addition, it was confirmed that isoflavonoids can be produced using a microorganism cotransformed with the gene encoding 2-hydroxyisoflavanone dehydratase and the gene encoding IFS.

Furthermore, the inventors of the present invention have found that the amino acid sequence of 2-hydroxyisoflavanone dehydratase contains a motif which is known to be present in carboxylesterases. These findings suggest that analogous proteins may be widely distributed over higher plants and a part of dehydration in the

biosynthesis of natural products may be mediated by this enzyme family.

Consequently, the present invention pertains to 2-hydroxyisoflavanone dehydratase contained in licorice, particularly to 2,7-dihydroxy-4′-methoxyisoflavanone-2,3-dehydratase (formononetin synthetic enzyme) and a nucleotide sequence encoding the same.

The 2-hydroxyisoflavanone dehydratase contained in licorice comprises a sequence of amino acids 1-328 which is represented by SEQ ID NO: 1. A cDNA sequence encoding the 2-hydroxyisoflavanone dehydratase is represented by SEQ ID NO: 2. Furthermore, the present invention also pertains to a recombinant DNA that expresses the novel gene and a transformant into which the gene is incorporated. The transformant is preferably yeast or *Escherichia coli*. The strain K12 of *E. coli* which is transfected with the gene is deposited as the Accession No. FERM P-19257 (Date of deposition: March 20, 2003) at the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, Tsukuba Central 6, 1-1 Higashi, Tsukuba, Ibaraki, Japan, followed by being transferred to the international deposition under the Budapest Treaty on March 15, 2004 as the Accession No. FERM BP-08662.

Furthermore, the present invention pertains to a method of producing isoflavonoids including isoflavones by a microorganism such as yeast , *E. coli* or a plant into which the gene is incorporated with or without a gene encoding IFS. The yeast preferably used for transformation is *Saccharomyces cerevisiae* strain BJ2168 (Nippon Gene Co., Ltd.). Besides, vectors for yeast transformation include pYES2 (Invitrogen Corporation), pESC-LEU (Stratagene), pESC-TRP (Stratagene), and pESC-HIS (Stratagene), etc.

The present invention also pertains to one of 2-hydroxyisoflavanone dehydratases in soybean, 2,7,4′-trihydroxyisoflavanone dehydratase (daidzein synthetic enzyme) and a nucleotide sequence encoding the same. The 2-hydroxyisoflavanone dehydratase in soybean is analogous to the 2-hydroxyisoflavanone dehydratase in licorice. However, the dehydratase in licorice uses a 4′-methoxy isoflavanone as a substrate, while the dehydratase in soybean uses a 4′-hydroxy isoflavanone as a substrate, and comprises a sequence of amino acids 1-319 represented by SEQ ID NO: 3. A cDNA sequence encoding the 2-hydroxyisoflavanone dehydratase in soybean is represented by SEQ ID NO: 4. Furthermore, the present invention also pertains to a recombinant DNA that expresses the novel gene and a transformant into which the gene is incorporated. The transformant is preferably yeast or *Escherichia coli*. The strain K12 of *E. coli* which is transfected with the gene is deposited as the Accession No. FERM

P-19256 (Date of deposition: March 20, 2003) at the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, Tsukuba Central 6, 1-1 Higashi, Tsukuba, Ibaraki, Japan, followed by being transferred to the international deposition under the Budapest Treaty on March 15, 2004 as the Accession No. FERM BP-08661.

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[Brief Description of the Drawings]

Fig. 1 is a diagram illustrating the production pathway from flavanone to isoflavonoids.

Fig. 2 is a diagram illustrating the cloning of cDNA that encodes 2-hydroxyisoflavanone dehydratase in licorice cells.

Fig. 3A shows the amino acid sequences of 2-hydroxyisoflavanone dehydratases in licorice and soybean.

Fig. 3B shows a molecular phylogenetical tree for the genes of leguminous plants, including licorice (*G. echinata* Dehydratase = licorice HIDM, Soybean TC98460 = soybean HIDH).

Fig. 4 is a HPLC profile of the product of 2-hydroxyisoflavanone dehydratase.

Fig. 5 shows the patterns of RT-PCR analysis, representing the respective gene-expression levels.

Fig. 6 shows HPLC chromatograms of extracts obtained by incubating the IFS and HIDH co-expression yeast (upper), the IFS expression yeast (middle), and the control yeast (lower) in naringenin-containing media, respectively.

[Best Mode for Carrying out the Invention]

Hereinafter, the present invention will be described concretely.

In the present specification, the 2-hydroxyisoflavanone dehydratase protein of licorice may be referred to as "HIDM", the gene encoding the protein may be referred to as "*HIDM*", the 2-hydroxyisoflavanone dehydratase protein of soybean may be referred to as "HIDH", and the gene encoding the protein may be referred

to as "*HIDH*".

The present invention pertains to 2-hydroxyisoflavanone dehydratase substantially having an amino acid sequence represented by SEQ ID NO: 1 or 3. As used herein, the phrase "which substantially has the amino acid sequence" means that the amino acid sequence includes ones having any amino acid mutation such as deletion, substitution, addition, and/or insertion as far as the amino acid sequence defines the activity of 2-hydroxyisoflavanone dehydratase. The number of amino acids to be involved in the deletion, substitution, addition, or insertion may be, for example, 1 to 20, preferably 1 to 10, and particularly 1 to 5. Particularly contemplated is, the amino acid sequence in which an amino acid residue is substituted with an amino acid having similar characteristics. The typical substitution occurs: among Ala, Val, Leu, and IIe; between Ser and Thr; between Asp and Glu; between Asn and Gln; between Lys and Arg; and between Phe and Tyr.

Furthermore, the present invention pertains to a polynucleotide substantially having the nucleotide sequence represented by SEQ ID NO: 2 or 4, or a nucleotide sequence complementary to the nucleotide sequence. In the present invention, the phrase "substantially having the nucleotide sequence" means that the nucleotide sequence includes a nucleotide sequence encoding 2-hydroxyisoflavanone dehydratase, and a nucleotide sequence having a difference from the sequence due to the nucleotide degeneracy

and having an appropriate additional sequences on the 5′-end, 3′-end, or both ends.

[Examples]

Hereinafter, the present invention will be concretely described with reference to the following examples. However, the present invention is not limited to the examples.

<Materials and Methods>

Materials and methods used in the present invention are as follows.

(1) Chemical materials

Daidzein, genistein, and biochanin A were obtained from Extrasynthese Co., Ltd., and (*RS*)-narigenin and *p*-nitrophenyl butyrate were obtained from Sigma Corporation. Formononetin was obtained from a stock in the inventors' laboratory.

Both 2,7,4′-trihydroxyisoflavanone (Non-Patent Document 13) and 2,7-dihydroxy-4′-methoxyisoflavanone (Non-Patent Document 12) were prepared as follows.

The 2,7,4′-trihydroxyisoflavanone was prepared by incubating a yeast microsome expressing CYP93C2 (IFS), liquiritigenin and NADPH, extracting with ethyl acetate, and separating by silica-gel TLC, followed by purification with reverse-phase HPLC. The 2,7-dihydroxy-4′-methoxyisoflavanone was prepared by extracting

a reaction mixture of *S*-adenosyl-*L*-methionine (SAM), 2,7,4′-trihydroxyisoflavanone, and recombinant 2,7,4′-trihydroxyisoflavanone 4′-*O*-methyl transferase (HI4′OMT) with ethyl acetate and then separating with silica gel TLC, followed by purification with HPLC.

2,5,7,4′-Tetrahydroxyisoflavanone was prepared by incubating yeast microsome expressing CYP93C2 (Non-Patent Document 1), (*RS*)-narigenin, and NADPH. The resulting product (Rf 0.30) was purified with silica gel thin-layer chromatography (TLC) [Kieselgel F254 (Merck Ltd.); solvent = toluene : ethyl acetate : methanol : petroleum ether = 6 : 4 : 1 : 3].

(2) Plant materials

Licorice cultured cells (strain AK-1) were established from the leaf and leaf stalk of *Glycyrrhiza echinata* in accordance with the document (Non-Patent Document 1). In a 1/2-concentrated Murashige-Skoog culture medium (solidified with 0.3%(w/v) gellan gum) containing α-napthalene acetic acid (1 μg/ml) and N6-benzyl adenine (1 μg/ml), the cells were cultured under 12-hour photoirradiation (6,000 luxes) and 12-hour darkness in cycles and then treated with elicitor to establish a cDNA library. The suspended culture was kept in the Murashige-Skoog culture medium supplemented with 2,4-dichlorophenoxyacetic acid (0.1 μg/ml) and kinetin (0.1 μg/ml) in dark place. The elicitor treatment was carried out using

a yeast extract (Invitrogen Corporation) at a concentration of 0.2% (w/v medium) (Non-Patent Document 14).

Seeds of soybean (*Glycine max* L. CV. Mikawashima : Tohoku Ltd.) were immersed in water for 24 hours and then seeded on a filter paper placed in a conical beaker. The soybean raised from seeds was grown for one week at room temperature under the conditions of 12-hour brightness and 12-hour darkness in cycles.

(3) Preparation of cell-free extract

All procedures were carried out at 4°C. Licorice cells (10 g) after elicitor treatment (for 24 hours) or 1-week old soybean raised from seeds (10 g) were homogenized in a mortar with 10 ml of a 100-mM potassium phosphate buffer (pH 7.5) containing 10% sucrose and 14 mM of 2-mercaptoethanol and with sea sand (2.5 g). The resulting homogenate was filtered through gauze and then centrifuged at 10,000 x g for 10 minutes. Supernatant was mixed with 2.5 g of Dowex 1-X2 (equilibrated with a 100-mM potassium phosphate buffer), followed by standing for 20 minutes. The solution obtained from the filtration was fractionated with ammonium sulfate and a 30-80% saturated fraction was then demineralized through a Sephadex G-25 column and dissolved in a 100-mM potassium phosphate buffer (pH 7.5) containing 10% sucrose and 14-mM 2-mercaptoethanol, followed by being used in the assay (approximately 600 µg/ml of protein).

(4) Assay of 2-hydroxyisoflavanone dehydratase

An enzyme preparation was added to 2,7,4′-trihydroxyisoflavanone, 2,7-dihydroxy-4′-methoxyisoflavanone, or 2,5,7,4′-tetrahydroxyisoflavanone (5 nmol each) in 2-methoxyethanol so as to be in a total volume of 100 µl, and then the whole was incubated at 30°C for 10 minutes.

The assay of 2,5,7-trihydroxy-4′-methoxyisoflavanone dehyderatase (biochanin A synthetase) was carried out as follows. 2,5,7,4′-tetrahydroxyisoflavanone (10 nmol) dissolved in 2-methoxyethanol was incubated together with licorice HI4′OMT (1 µg) and 1µmol of *S*-adenosyl-L-methionine (SAM) at 30°C for 15 minutes.

A concentrated ethyl acetate extract was incubated with recombinant licorice HIDM (1 µg) at 30°C for 10 minutes. An ethyl acetate extract from the mixture was analyzed by high-performance liquid chromatography (HPLC). The HPLC for both daidzein and formononetin analysis was carried out using a Capcell pak C18 MG column (4.6 x 150 mm; Shiseido Co., Ltd.) at 40°C (flow volume = 0.8 ml/min) (Non-Patent Document 12) with a linear gradient elution for 40 minutes from 35% to 55% methanol in 3% aqueous acetic acid. 5-Hydroxyisoflavones were analyzed using a Capcell pak C18 MG column (4.6 x 150 mm; Shiseido Co., Ltd.) with a 50% methanol aqueous solution

(for genistein) or a 55% methanol aqueous solution (for biochanin A) at 40°C (flow rate = 0.8 ml/min).

The purified recombinant protein (approximately 10 ng of protein) and cell-free extracts of licorice and soybean (approximately 10 μg of protein) were used to determine their specific activity. The concentration of isoflavone was calculated from the HPL peak area of the standard sample of each of daidzein, formononetin, and genistein.

(5) Cloning of cDNA (*HIDM*) encoding 2,7-dihydroxy-4'-methoxyisoflavanone dehydratase of licorice cells

The expression of protein and the preparation of a crude extract of *E. coli* were carried out as described in a previous document (Non-Patent Document 12).

A cDNA expression library constructed from licorice cells subjected to an elicitor treatment with a yeast extract (6 or 12 hours) (Non-Patent Document 12) was used for screening.

The licorice λZapII cDNA library was converted into a phagemid from by *in vivo* excion using Exassist helper phage (Stratagene Corporation) and *E. coli* DH5α F' IQ (Invitrogen Corporation).

Phagemid was introduced into DH5α F' IQ and then *E. coli* cells proliferated on an agar plate with Luria-Bertani (LB) / ampicillin (50 μg/ml). From a mother plate, five independent cDNA fraction

pools were prepared, each of which contained approximately 30,000 *E. coli* transformants in LB/ampicillicin (50 μg/ml) culture medium. Subsequently, the cells were incubated in a LB-liquid medium containing 5 mM of IPTG and then collected, followed by preparing a crude enzyme solution.

Recombinant licorice HI4′OMT (50 ng) was added to 2,7,4′-trihydroxyisoflavanone (0.4 nmol) dissolved in 2 μl of 2-methoxyethanol (Non-Patent Document 12) and then preincubated in the presence of 0.4 nmol of S-adenosyl-L-[methyl-^{14}C] methionine ([^{14}C] SAM, 2.26 GBq/mmol, Amersham Biosciences Corporation) at 30°C for 3 minutes (total volume = 50 μl). Subsequently, the crude extract of *E. coli* pool (100 μl) was added to the mixture and then the whole was further incubated at 30°C for 10 minutes. The reaction was terminated by the addition of ethyl acetate, the ethyl acetate extract of the mixture was developed through silica gel TLC [LK6DF (Whatman Ltd.); solvent used was chloroform : acetone : 25% ammonium solution = 70 : 29 : 1 ; 2, 7-dihydroxy-4′-methoxyisoflavanone (Rf 0.15), formononetin (Rf 0.30)], and then analyzed by an image analyzer (Typhoon 8600, Amersham Biosciences Corporation). A positive pool, which generated [^{14}C] formononetin, was selected for a subsequent screening and divided into ten small-sized pools (approximately 3,000 clones/pool). The fractionation of the positive pool and assay were repeated four times to isolate a clone (*HIDM*) showing 2,7-dihydroxy-4′-methoxyisoflavanone dehydratase activity (see

Fig. 2).

The plasmid was collected, and a nucleotide sequence was then determined using an auto-sequencer (LIC-4000, Aloka Co., Ltd.).

(6) Cloning of soybean cDNA homologous to *HIDM*

Poly(A)+RNA was isolated from soybean raised from seeds using RNeasy Plant Mini Kit (Qiagen Ltd.) and cDNA was then synthesized using Ready-To-Go T-Primed First Strand Kit (Amersham Biosciences Corporation).

Two PCR-specific primers containing a *Nde*I or *BamH*1 site (underlined part) were designed from a coding region of TC98460 of soybean EST, which designated initiation and termination codon sequences (TC98-Fow, GTCATATGGCGAAGGAGATAGTGAA (SEQ ID NO: 5); TC98-Rev, AGGGATCCATCAAACCAGAAAAGA (SEQ ID NO: 6)). The cDNA (*HIDH*) obtained by the reverse transcription (RT)-PCR with these primers using soybean cDNA as template was cloned into a pT7Blue T-vector (Novagen Ltd.) to determine its nucleotide sequence.

(7) Heterologous expression of licorice *HIDM* and soybean *HIDH* in *E. coli*

Two primers containing a *Nde*I or *BamH*1 site (underlined part) were designed from a coding region of the licorice HIDM (GeDchy-F, GTCATATGGCTTCTTCAACCTCAAC (SEQ ID NO: 7) GeDehy-R, CTGGATCCTCAAACAAGGAAGGAAG (SEQ ID NO: 8)).

The *Nde*I-*BamH*1 fragment of the PCR product from licorice *HIDM* was cloned into the corresponding sites of pET28a (Novagen Ltd.). In addition, the *Nde*I-*BamH*1 fragment of soybean cDNA (*HIDH*) was also subcloned into the corresponding sites of the pET28a. The expression and purification of recombinant 2-hydroxyisoflavanone dehydratases were carried out by the following procedures (Non-Patent Document 12). The *E. coli* BL21 (DE3) cells transformed with the vector containing HIDM or HIDH were incubated up to OD600 ≒ 0.4 in a LB/ampicillin medium supplemented with 20 ml of 50 μg/ml kanamycin or ampicillin at 30°C. IPTG was added to the culture so as to be a final concentration of 1.0 mM and the whole was incubated at 30°C for six hours. The licorice HIDM and soybean HIDH were purified from the crude extracts of *HIDM* and *HIDH*-expression *E. coli* using HisTrap Kit (Amersham Biosciences Corporation), respectively.

(8) Preparation of recombinant yeast by which 2-hydroxyisoflavanone synthase (IFS) and 2-hydroxyisoflavanone dehydratase are co-expressed

Vector (pYES-CYP93C2) used for the expression of IFS (CYP93C2) of licorice in yeast was those described in Non-Patent Document 1 and Patent Document 1 (the coding region of the CYP93C2 gene was clond into the *Kpn*I and *EcoR*I sites downstream of a galactose induction promoter GAL1 of a yeast expression vector (pYES2, Invitrogen Corporation)).

The vector (pESC-HIDE) for the expression of soybean 2-hydroxyisoflavanone dehydratase (HIDH) was prepared as follows. Two different primers added with *Apa*I and *Xho*I sites (underlined portions) [HIDH-F1 (5′ - G<u>GGGCCC</u>GGATCCATGGCGAAGGAGATAGTGAAAG-3' (SEQ ID NO: 9)) and HIDH-R1 (5′ -GG<u>GAGCTC</u>GAGTCAAACCAGAAAAGAAGCC-3' (SEQ ID NO: 10)] were designed from the coding region of soybean 2-hydroxyisoflavanone dehydratase (HIDH).

The primers and KOD polymerase (Toyobo Co., Ltd.), as well as soybean HIDH cDNA as a template, were subjected to a PCR (98°C for 15 sec, 60°C for 15 sec, and 74°C for 30 sec in 15 cycles). The PCR product was treated with *Apa*I and *Xho*I and then inserted into *Apa*I and *Xho*I sites downstream of galactose induction promoter GAL1 of an yeast expression vector (pESC-Leu, Stratagene) to produce pESC-HIDH.

The yeast, *Saccharomyces cerevisiae* strain BJ2168 (a; prc1-3407, prb1-1122, pep4-3, leu2, trp1, ura3-511) (Nippon Gene Co., Ltd.), was transformed using an electroporation device (Cellject Duo, Thermo Electron Corporation). The electropolartion was carried out according to the procedures recommended by Thermo Electron Corporation. The transformants were selected in a culture medium containing a yeast nitrogen base without amino acids (6.7 g/l, Invitrogen Corporation), glucose (20 g/l), tryptophan (20 mg/l), and agar (20 g/l). The following three recombinant yeasts were prepared: (1) control yeast (pYES2 and

pESC-Leu were introduced into strain BJ2168), (2) IFS expression yeast (pYES-CYP93C2 and pESC-Leu were introduced into strain BJ2168), and (3) IFS and 2-hydroxyisoflavanone dehydratase co-expression yeast (pYES-CYP93C2 and pESC-HIDH were introduced into strain BJ2168).

(9) Determination of carboxylesterase activity

The specific carboxylesterase activities of recombinant licorice HIDM and soybean HIDH proteins were calculated from the generation rate of *p*-nitrophenol measured at 30°C by an absorbance at 400 nm in 1.5 ml of a 50-mM Tris-HCl buffer containing 150 mM of NaCl and 750 nmol of *p*-nitrophenyl butyrate (Heymann 1981). Commercially-available pig liver carboxylesterase (Sigma Corporation) was used as a positive control. Thermally-denatured (100°C, 10 minutes) licorice HIDM, soybean HIDH, and pig liver carboxylesterase protein were analyzed as negative controls, respectively.

(10) RT-PCR analysis

Suspension-cultured licorice cells were treated with yeast extract (Invitrogen Corporation) and then harvested at time periods of 3, 6, 12, 24, and 48 hours after treatment (Non-Patent Document 1). In addition, mRNA was extracted using a Straight A's mRNA isolation system (Novagen Ltd.) to synthesize cDNA. For the RT-PCR,

specific primers designed from licorice *HIDM*, *IFS* (Non-Patent Document 12) and *HI4'OMT* (Non-Patent Document 12) were used. The reaction was initiated by denaturation at 94°C for one minute and then three incubation steps (94°C for 1 min; 55°C for 1 min; and 72°C for 1 min) were repeated for 30 cycles. The product was subjected to 1.2%(w/v) agarose gel electophoresis and then stained with ethidium bromide.

The results of the present invention obtained by the above materials and methods will be described below.

(1) 2-hydroxyisoflavanone dehydratase activities in licorice cells and soybean raised from seeds

After the elicitor treatment, the licorice cells accumulate medicarpin (a 4'-methoxyisoflavonoid, see Fig. 1) but not 4'-hydroxyisoflavonoids (Nakamura et al. 1999). Soybeans are known to produce glycoconjugates of 4'-hydroxylated isoflavones, daidzein, genistein and glycitein (Dewick 1986, Dewick 1993, and Aussenac 1998). Therefore, it seems that the extracts from licorice cells and soybean raised from seeds have activities to produce formononetin and daidzein from 2-hydroxyisoflavonones with appropriate substitutions, respectively. The results obtained by investigating those activities using HPLC are shown in Table 1.

Table 1

Specific activities of 2-hydroxyisoflavanone dehydratase and carboxylesterase in the crude extracts and recombinant proteins of licorice and soybean

Substrate	Product	Specific activity (a), (b)				
		Licorice		Soybean		Carboxyl-esterase from pig liver (nkatal/mg)
		Crude extract (c) (pkatal/mg)	Recombinant protein (d) (nkatal/mg)	Crude extract (c) (pkatal/mg)	Recombinant protein (d) (nkatal/mg)	
2,7-dihydroxy-4′-methoxyisoflava-none	formononetin	123.0±8	52.1±1.5	97.4±10	0.90±0.1	-(e)
2,5,7,4′-tetrahy-droxyisoflavanone	genistein	-(e)	-(e)	19.6±3	3.55±0.6	-(e)
2,7,4′-trihydroxy-isoflavanone	daidzein	0.8±0.03	0.70±0.08	197.3±15	43.6±4	0
p-nitrophenyl butyrate	*p*-nitrophenol	-(e)	0.32±0.07(f)	-(e)	7.20±0.4(f)	425±7(f)

(a) Mean ± SD from three independent experiments.

(b) Specific activities were calculated from the rate of isoflavone and *p*-nitrophenol production.

(c) Ammonium sulfate (30% to 80% saturation) precipitate of the cell-free extract of licorice cells (strain AK-1) elicited with 0.2% yeast extract for 24 hours or the cell-free extract of soybean seedling.

(d) Purified recombinant proteins were used for the assays.

(e) Unanalyzed.

(f) No hydrolyzing activity was detected in assays with heat-treated (100°C, 10 minutes) proteins and *p*-nitrophenyl butyrate.

As is evident from Table 1, when 2,7-dihydroxy-4′-methoxyisoflavanone was incubated with the cell-free extract of licorice, formononetin was produced. A small amount of daidzein produced from 2,7,4′-trihydroxyisoflavanone with the licorice extract was observed but the activity thereof was approximately 160 times as small as the production of formononetin.

In the case of the cell-free extract from soybean raised from seeds, it was detected that formononetin and daidzein were produced at a ratio of roughly 1:2 from 2,7-dihydroxy-4′-methoxyisoflavanone (from which formononetin is produced) and 2,7,4′-trihydroxyisofravonone (from which daidzein is produced). Furthermore, the soybean extract catalyzed the production of genistein from 2,5,7,4′-tetrahydroxyisoflavanone at a level of approximately 1/10 with respect to the production of daidzein.

On the other hand, spontaneous dehydration of 2-hydroxyisoflavanone could be ignored under experimental conditions in which the concentration of a substrate was 50 μM in a neutral buffer (pH 7.5). These results strongly suggest that the dehydration of 2-hydroxyisoflavanone which forms isoflavone in plant cells is an enzyme-catalyzed reaction. Furthermore, it seems that licorice and soybean have different substrate specificities with respect to 2-hydroxyisoflavanone dehydratase.

(2) The preparation of 2-hydroxyisoflavanone dehydratase cDNA of

licorice by functional expression fractionation screening.

The high sensitive and the specific detection of the conversion from 2,7-dihydroxy-4'-methoxyisoflavanone to formononetin was made possible using the purified 2,7,4'-trihydroxyisoflavanone prepared by recombinant IFS in yeast (Non-Patent Document 1), [^{14}C]-SAM, and affinity-purified recombinant licorice HI4'OMT having 6 histidine residues at the N-terminus (Non-Patent Document 12) to produce [^{14}C]-2,7-dihydroxy-4'-methoxyisoflavanone prior to the assay. The screening of cDNA of the dehydratase was carried out using a cDNA expression library prepared from licorice cells treated with yeast extract (Non-Patent Document 12). The initial screening was performed on five cDNA pools (30,000 transformants/pool). The extract from the *E. coli* pool was analyzed such that it was allowed to react with the above mixture which produced [^{14}C]-2,7-dihydroxy-4'-methoxyisoflavanone and then an ethyl acetate extract from the reaction mixture was analyzed by TLC autoradiography. It was found that two pools produced [^{14}C] formononetin. One of the positive pools optionally chosen was fractionated into ten small-sized pools (approximately 3,000 clones/pool). The expression and analysis of protein were carried out again, and a positive pool was then identified from 10 pools. The positive pool was repeatedly fractionated and analyzed. One positive pool out of the 10 pools was repeatedly identified from third (approximately 300 clones/pool), fourth (30 clones/pool),

and fifth (3 clones/pool) screenings. Finally, a single *E. coli* clone representing 2,7-dihydroxy-4'-methoxyisoflavanone dehydratase activity was isolated (see Fig. 2).

The cDNA encoding the enzyme was collected, and then the sequence was determinated sequence using a sequencer. The cDNA of HIDM (2-hydroxyisoflavanone dehydratase methoxy type) has a 1,178-bp nucleotide and encoded 328 amino acids (Fig. 3A). The search for protein-protein BLAST (http: //www.ncbi.nlm.nih.gov/BLAST/) revealed that an estimated amino acid sequence of licorice HIDM indicates 40% identity with putative proteins of *Arabidopsis thaliana* (Accession NOS: At1g47480, AT3g48690, and At3g48690), 34% identity with *Nicotiana tabacum* hsr203J (Accession NO: X77136) (Pontier et al., 1994), 31% identity with pea E86 (Accession NO: AB026296) (Ichinose et al., 2001), and 32% identity with carboxylesterase of *Archaeoglobus fulgidus* (thermophilic sulfur bacteria) (Accession NO: 1JJIA) (Manco et al., 2000). In addition, the licorice HIDM had the motif of a conserved sequence recorded for carboxylesterases (approximately 40 to 180 amino acids from the N-terminal).

Conserved sequences (His85-Gly86-Gly87, a boxed sequence in Fig. 3A) shared with lipases and esterases to form an oxy anion hole were present in the motif of carboxylesterase (Contreras et al., 1996, Laurell et al., 2000, and Hosokawa 2000). In the licorice HIDM protein, even though the Ser residue stored in catalytic triads

in typical lipase and esterase (Osterlund et al., 1996, Contreras et al., 1996, Manco et al., 2000, and Hosokawa 2002) was substituted by a Thr residue, it was recognized that a hypothetical catalytic triad (Thr 173, Asp 272, and His 304) is found on the outside of the motif of carboxylesterase. In Fig. 3A, the hypothetical catalytic triad (Thr 173, Asp 272, and His 304) is denoted by an asterisk (*).

(3) Search of homologous cDNA of licorice dehydratase in cDNA libraries of leguminous plants

By retrieving from the expressed sequence tags (ESTs) of soybean (http://www.tigr.org/tdb/tgi/gmgi/), *Medicago truncatula* (http://www.tigr.org/tdb/tgi/mtgi/), and *Lotus japonicus* (http://www.kazusa.or.jp/cn/plant/lotus/EST/) (Asamizu et al., 2000), it was revealed that these plants had cDNAs which indicated identity with the licorice HIDM (amino acid identity > 50%). However, those sequences have been annotated as hypothetical proteins. A molecular phylogenetical tree represented that soybean BM177194, *L. japonicus* TC3332, and *M. truncatula* TC43540 proteins form the same branch as that of the dehydratase of licorice (amino acid identity > 80%) (Fig. 3B). The soybean BG456496 has > 60% identity as those of the four proteins, and form the other branch with *M. truncatula* BG456496 (Fig. 3B).

(4) Characterization of recombinant 2-hydroxyisoflavanone dehydratases of licorice and soybean

The EST sequence TC98460 of soybean has predicated initiation and terminal codons. The coding region of cDNA was cloned from soybean seedlings by RT-PCR and designated as *HIDH* (2-hydroxyisoflavanone dehydratase hydroxy type).

Licorice *HIDM* and soybean *HIDH* were expressed in *E. coli* and recombinant proteins having six histidine residues at the N-terminal were then purified, followed by measuring the activity thereof to 2-hydroxyisoflavanones. As shown in Fig. 4A, the incubation of licorice HIDM with 2,7-dihydroxy-4′-methoxyisoflavanone led to the production of formononetin. The product (formononetin) can be identified and confirmed by making a comparison between the Rt value thereof and the Rt value of a standard sample, as well as carrying out electron impact mass spectrometry (molecular ion peak of m/z 268, and retro-Diels-Alder fragment peak of m/z 132). Furthermore, a small amount of daidzein was produced from 2,7,4'-trihydroxyisoflavanone by licorice HIDM. The specific activity of licorice HIDM to 2,7-dihydroxy-4'-methoxyisoflavanone is 74 times as high as one to 2,7,4'-trihydroxyisoflavanone and shows that the biochemical characteristics of the recombinant protein may correspond to those of a licorice cell-free extract (Table 1).

When the recombinant soybean HIDH was assayed using 2,7,4′-

trihydroxyisoflavanone and 2,7-dihydroxy-4'-methoxy isoflavanone, it was confirmed that the respective peaks of daidzein and formononetin emerged on HPLC (Fig. 4A). The chemical structures of isoflavones were reconfirmed by the electron impact mass spectrometry. Furthermore, the soybean HIDH catalyzed the production of genisein from 2,5,7,4'-trihydroxyisoflavanone (Fig. 4A). As shown in Table 1, the specific activity of HIDH was the highest with respect to 2,7,4'-trihydroxyisoflavanone but comparatively low (approximately 1/10) with respect to another 4'-hydroxylated substrate and extremely low with respect to another substrate having a 4'-methoxy group.

Furthermore, when a compound expected to be 2,5,7-trihydroxy-4'-methoxyisoflavanone, which was obtained by incubating the recombinant licorice HI4'OMT with 2,5,7,4'-tetrahydroxyisoflavanone and SAM, was incubated with the licorice HIDM, biochanin A was detected by HPLC (Fig. 4B).

(5) Carboxylesterase activity of recombinant licorice and soybean 2-hydroxyisoflavanone dehydratases

In the carboxylesterase assay, *p*-nitrophenyl butyrate is a substrate commonly used. The recombinant licorice and soybean dehydratases displayed weak activity against *p*-nitrophenyl butyrate (Table 1). On the other hand, carboxylesterase from pig lever did not dehydrate 2,7,4'-trihydroxyisoflavanone (Table 1).

(6) Gene expression in formononetin pathway in licorice cells

The present RT-PCR analysis revealed that transcription levels of *HIDM*, *HI4'OMT*, and *IFS* of licorice cells increased at 6 to 12 hours after the treatment with yeast extracts, respectively (Fig. 5).

(7) Isoflavone production in recombinant yeast where 2-hydroxyisoflavanone synthase (IFS) and 2-hydroxyisoflavanone dehydratase were co-expressed

Three different recombinant yeasts were compared with respect to their abilities to produce isoflavone. Here, those yeasts were those described in the previous section for the preparation of recombinant yeast in which 2-hydroxyisoflavanone synthase (IFS) and 2-hydroxyisoflavanone dehydratase of soybean (HIDH) were co-expressed. That is, the yeasts were (1) control yeast (pYES2 and pESC-Leu were introduced into strain BJ2168), (2) IFS expression yeast (pYES-CYP93C2 and pESC-Leu were introduced into strain BJ2168), and (3) IFS and HIDH co-expression yeast (pYES-CYP93C2 and pESC-HIDH were introduced into strain BJ2168).

Each of those three different yeasts was incubated overnight (28°C) with shaking in a 1.5-ml minimum liquid culture medium[yeast nitrogen base without amino acids (6.7 g/l), glucose (20 g/l), tryptophan (20 mg/l)]. Then, the yeast cells were collected by

centrifugation and then the yeast cells were suspended in a 3-ml YPG liquid medium[yeast extract (10 g/l), peptone (20 g/l), galactose (20 g/l)], and incubated overnight to induce protein expression.

Then, the yeast cells were collected by centrifugation and then the yeast cells were suspended in a 0.5-ml YPG liquid medium containing 50 μg of naringenin (dissolved in 5 μl tween 80 and 5μl ethanol) and then incubated overnight. Glass beads were added to the culture medium to crush the cells, followed by extracting with ethyl acetate. The extract was dried and then dissolved in methanol, followed by analysis on HPLC [column: CAPCELL PAK C18 MG column (4.6 x 150 mm, Shiseido Co., Ltd.); 40°C; 0.8 ml/min; solvent, which is linear gradient so as to be 30% methanol (0 min) to 50% methanol (30 min)]. Based on a peak area of genistein preparation, the amount of genistein in each sample was obtained.

As a result, for each of the recombinant yeast (3) in which IFS and HIDH were co-expressed and the recombinant yeast (2) in which IFS was expressed by itself, the production of genistein and 2,5,7,4'-tetrahydroxyisoflavanone was confirmed on HPLC. For the control yeast (1), the production of either or both compounds could not be observed. The yeast (3) where IFS and HIDH were co-expressed produced 3.2 ± 0.2 μg of genistein (average of three experiments). The recombinant yeast, where IFS was expressed by itself produced 0.8 ± 0.1 μg of genistein (average of three experiments). Thus, it was found that the amount of isoflavone produced increased when

IFS and HIDH were co-expressed (see Fig. 6).

From the above results, the followings become evident.

In the present invention, cDNA, which encodes 2-hydroxyisoflavanone dehydratase, licorice *HIDM*, and soybean *HIDH* were cloned, respectively.

HIDM and HIDH show different substrate specificity to 2-hydroxyisoflavanone having 4′-methoxyl and 4′-hydroxyl substituents. These enzymes can be referred to as 2,7-dihydroxy-4'-methoxyisoflavanone 2,3-dehydratase (formononetin synthetic enzyme) of licorice and 2,7,4'-trihydroxyisoflavanone 2,3-dehydratase (daidzein synthetic enzyme) of soybean employing the most preferred substrate of each enzyme into the nomenclature. It is important that the substrate specificity can be reflected in the structure of isoflavone contained in each of plant species (and furthermore, isoflavonoid on the downstream of the biosynthetic pathway). Therefore, there is an extremely high probability that the production of isoflavone from 2-hydroxyisoflavanone in plant cells is enzyme-dependent.

The specific activity of 2-hydroxyisoflavanone dehydratase reaction by recombinant licorice HIDM is roughly 400 to 900 times higher than that of the crude extract of licorice. Thus, any of the gene recombinant protein and the crude extract showed an extremely higher selectivity to 2,7-dihydroxy-4′-methoxyisoflavanone than to

2,7,4′-trihydroxyisoflavanone (see Table 1). It strongly suggests that the main activity of the crude extract may be found in the HIDM protein. Furthermore, as the HIDM mRNA of the induced licorice cells equally accumulate both IFS and HI4′OMT, it is suggested that HIDM may participate in the biosynthesis of formononetin.

On the other hand, the soybean extract catalyzed the dehydration activities against 2,7-dihydroxy-4′-methoxyisoflavanone and 2,7,4′-trihydroxyisoflavanone at a ratio of approximately 1 : 2. In contrast, the dehydratase activity of recombinant soybean HIDH protein was very specific to a 4′-hdyroxylated 2-hydroxyisoflavanone (see Table 1). Consequently, there is a high possibility that HIDH may cause the production of 4′-hydroxylated isoflavone from soybean.

In the present invention, as for a more interesting finding, 2-hydroxyisoflavanone dehydratase is a protein having a sequence which can be classified as carboxylesterase of hydrolase family. Actually, the soybean HIDH had a weak carboxylesterase activity against *p*-nitrophenyl butyrate (approximately 1/50 of the pig lever enzyme). The present invention has originally demonstrated that the protein in this family may catalyze dehydration.

The characteristic features of 2-hydroxyisoflavanone dehydratase, which was reported with respect to *P. lobata*, correspond to those of the soybean HIDH (Hakamatsuka et al., 1998). The protein of *P. lobata* has a molecular weight of 38 kDa, which approximates

a calculated value of the soybean HIDH (35,115). Furthermore, the specific activity (56.8 mkatal/kg protein) of dehydratase of *P. lobata* against 2,7,4′-trihydroxyisoflavanone is almost equal to the activity (43.6 nkatal/mg) of the gene recombinant soybean. Very interestingly, the His residue in the protein of *P. lobata* has been reported very important for the activity of the protein (Hakamatsuka et al., 1998). The His is one of the amino acids in the catalytic triad of carboxylesterase (Satoh and Hosokawa 1995 and Wei et al., 1999). Therefore, 2-hydroxyisoflavanone dehydratase of *P. lobata* may also be a protein in the carboxylesterase family.

Some dehydratase genes / proteins have their own characteristic features which have been determined from several kinds of plants. Those include dehydroquinate dehydratase (Deka et al., 1994), δ-aminolevulinate dehydratase (Kaczor et al., 1994), imidazole glycerol phosphate dehydratase (Tada et al., 1994), and allene oxide synthetic enzyme (Song et al., 1993). However, between those dehydratases and HIDM/HIDH, no significant homology of nucleotide and amino acid sequences can be found.

Proteins having a carboxylesterase motif which is homologous to HIDM/HIDH to some extent are widely distributed to the plant kingdom. In addition, for biosynthetically producing vegetable natural products, there are many dehydration reactions in which the characteristics of enzymes have not been defined. For instance, there is a dehydrative ring-formation of 2'-hydroxyisoflavan-4-ol

to form pterocarpan skeleton (Bless and Barz 1988, Guo et al., 1994a, and Guo et al., 1994b). Experiments with the microsome reveals that P450s are responsible for the production of methylene ring and the cyclization of a prenyl substituent on phenol ring during the biosynthesis of isoflavonoids in chickpea and soybean (Clemens and Barz 1996, Welle and Grisebach 1988). However, dehydratase may contribute to those reactions together with P450, and the enzyme protein may be classified in the carboxylesterase family. In addition, it is reported that several plant genes encoding this type of proteins to which no catalytic functions are assigned have been induced in response to pathogenesis (Pontier et al., 1994, Walden et al., 1999, Ichinose et al., 2001, Tronchet et al., 2001, and Benzier et al., 2002). They may be dehydratases which participate in the biosynthesis of defence compounds.

It is very important to identify a 2-hydroxyisoflavanone dehydratase gene for metabolic engineering of leguminous and non-leguminous plants. So far, for the production of isoflavonoid in non-leguminous plants, transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* over expressing soybean IFS have been constructed (Non-Patent Document 3, Non-Patent Document 7, and Non-Patent Document 3). However, the productivity of isoflavonoid in the transformants, was not satisfactory. In a typical case, genistein can be produced approximately 2 to 4 ng per gram fresh weight of *Arabidopsis thaliana* (Non-Patent Document 7, Non-Patent Document

8). On the other hand, isoflavone can be produced approximately 4 to 10 mg per gram dry weight of soybean seeds (Aussenac et al., 1998) and isoflavone can be produced approximately 3 mg per gram fresh weight of lupin seedling (Katagiri et al., 2000). Competition between a metabolic flow from flavanone to 2-hydroxyisoflavanone and another flow to 3-hydroxyflavanone may be a bottle neck for the production of isoflavone in transgenic *Arabidopsis thaliana*, and, indeed, the isoflavone production in A. thaliana flavanone 3-hydroxylase mutant transformed with IFS increased to 6- to 31-folds (Liu et al., 2002).

Isoflavone production in a recombinant yeast coexpressing IFS and HIDH were higher that in the recombinant yeast expressiong IFS. Thus, the amount of isoflavone produced can be expected to increase when IFS and HIDH are coexpressed in non-leguminous plants.

Furthermore, it is feasible to engineer an isoflavonoid pathway of a leguminous plant by genetic engineering using *HIDH* and *HIDM* as transgenes.

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[Industrial Applicability]

According to the present invention, as described above, dehydrate which plays an important role in the production step of isoflavone in a plant body is isolated, so that an amino acid sequence thereof and novel polynucleotides encoding such a sequence can be provided. Furthermore, the present invention allows the use of the gene thus obtained to the production of isoflavonoid including isoflavone.

[Reference to deposited biological material]

(1) (a) Name and address of the depository institution to which the biological material has been deposited

Name: International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (AIST)

Address: Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan

(b) Date of deposition to the institution (a)

March 20, 2003 (original deposition date)

March 15, 2004 (date of transfer to the deposition based on Budapest Treaty)

(c) Accession number that the institution (a) provides for the deposition

FERM BP-08661

(2) (a) Name and address of the depository institution to which the

biological material has been deposited

Name: International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (AIST)

Address: Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan

(b) Date of deposition to the institution (a)

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